



(19) World Intellectual Property Organization International Bureau



(43) International Publication Date 31 December 2003 (31.12.2003)

(10) International Publication Number WO 2004/001042 A2

(51) International Patent Classification 7: C12N 15/10

(61) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CZ, CA, CH, CN, CO, CR, CU, CZ, DK, DM, DZ, EC, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MX, MZ, NO, NZ, OM, PH, PT, RO, RU, SC, SD, SE, SG, SK, SI, TL, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(30) Priority Data: PCT/DK02/00419 20 June 2002 (20.06.2002) DK 10/175,539 20 June 2002 (20.06.2002) US PA 2002 01563 19 December 2002 (19.12.2002) DK 60/434,664 20 December 2002 (20.12.2002) US

(71) Applicant (for all designated States except US): NUEVO LUTION A/S (DK/DK); Renegade 8, 5, DK-2100 København Ø (DK).

(72) Inventors; and (73) Inventors/Applicants (for US only): FRESKGÅRD, Per-Ola [SE/SE]; Ringvägen 7, S-25 93 Vellinge (SE).

(74) Title: MICROARRAYS DISPLAYING ENCODED MOLECULES

(54) Title: MICROARRAYS DISPLAYING ENCODED MOLECULES

(57) Abstract: Disclosed is a microarray comprising a plurality of single stranded nucleic acid probes immobilized in discrete areas of a solid support, said probes being hybridized to a library of complexes, wherein each complex comprises an encoded molecule and a template which codes for said molecule, said template comprising a number of codons which codes for chemical entities which upon reaction form a reaction product which at least partly form part of the encoded molecule.

Microarrays displaying encoded molecules

Technical Field of the Invention

The present invention relates to an oligonucleotide microarray which has been annealed to a complex so as to present at spatially defined spots encoded molecules.

Background

Microarrays have found a wide acceptance in various analysis concepts. Microarrays can be used to profile gene patterns yielding information on the genotype of an individual. Information on the expression of the genes may be accomplished using messenger RNA (mRNA) samples in order to obtain knowledge on a disease state, hormone action, infection etc. The presence or absence of a particular SNP may also be verified using Microarrays.

15

In US 60207,446 B1 it has been suggested to use a microarray to present polypeptides linked to the mRNA which is responsible for the formation thereof. The mRNA-protein fusion is formed by allowing a ribosome to translate an mRNA provided at the 3' end with puromycin and linking the formed protein to said mRNA at the termination of the translation. By annealing a mixture of RNA-protein fusions to a microarray it is possible to display the proteins. As spots on the microarray comprise different sequences of nucleotides and the RNA sequences anneal sequence specifically to the probes, the proteins are presented at spatially defined areas of the microarray.

25

The prior art is restricted to the presenting of proteins on a microarray. According to an object of the present invention it is desired to expand the type of molecules which can be presented on a microarray. In one aspect of the invention, it is small molecules which are presented and in another aspect it is unnatural polymers that are presented. Notably, the present invention is not limited to the reaction products of the 20 naturally occurring amino acids, which allow for a higher diversity of the presented molecule and the possibil-

WO 2004/001042 A2

(54) Title: MICROARRAYS DISPLAYING ENCODED MOLECULES

(57) Abstract: Disclosed is a microarray comprising a plurality of single stranded nucleic acid probes immobilized in discrete areas of a solid support, said probes being hybridized to a library of complexes, wherein each complex comprises an encoded molecule and a template which codes for said molecule, said template comprising a number of codons which codes for chemical entities which upon reaction form a reaction product which at least partly form part of the encoded molecule.

4

Each of the complexes hybridised to the microarray comprises an encoded molecule and a template which codes for the encoded molecule. Usually, the template comprises a nucleic acid, such as an oligonucleotide. The oligonucleotide can contain any of the nucleotides mentioned below.

It is preferred that the template is divided into coding regions or codons which codes for specific chemical entities. A codon is a sequence of nucleotides or a single nucleotide. The nucleotides are usually amplifiable and the nucleobases are selected from the natural nucleobases (adenine, guanine, uracil, thymine, and cytosine) and the backbone is selected from DNA and RNA, preferably DNA.

The codon may be a single nucleotide. In the generation of a library, this will allow for the incorporation of four different chemical entities into the encoded molecule. However, to obtain a higher diversity a codon in certain embodiments preferably comprises at least two and more preferred at least three nucleotides. Theoretically, this will provide for 4² and 4³, respectively, different chemical entities. The codons will usually not comprise more than 100 nucleotides. It is preferred to have codons with a sequence of 3 to 30 nucleotides.

15

The template will in general have at least two codons, which are arranged in sequence, i.e. next to each other. Each of the codons may be separated by a spacer group. Depending on the encoded molecule formed, the template may comprise further codons, such as 3, 4, 5, or more codons. Each of the further codons may be separated by a suitable spacer group. Preferably, all or at least a majority of the codons of the template are arranged in sequence and each of the codons is separated from a neighbouring codon by a spacer group. Alternatively, codons on the template may be designed with overlapping sequences.

5

Generally, it is preferred to have more than two codons on the template to allow for the synthesis of more diverse template-directed molecules. In a preferred aspect of the invention the number of codons of the template is 2 to 100. Still more preferred is templates comprising 3 to 20 codons.

5 The spacer sequence may serve various purposes. In one setup of the invention, the spacer group identifies the position of a codon. Usually, the spacer group either upstream or downstream of a codon comprises information which allows determination of the position of the codon. The spacer group may also or in addition provide for a region of high affinity. The high affinity region will ensure that the hybridisation of the template with the anti-codon will occur in frame. Moreover, the spacer sequence may adjust the annealing temperature to a desired level.

A spacer sequence with high affinity can be provided by incorporation of one or more nucleobases forming three hydrogen bonds to a cognate nucleobase. An example of a nucleobase having this property is guanine. Alternatively, or in addition, the spacer sequence may be subjected to back bone modification. Several back bone modifications provides for higher affinity, such as 2'-O-methyl substitution of the ribose moiety, peptide nucleic acids (PNA), and 2'-O-methylene cyclisation of the ribose moiety, also referred to as LNA (Locked Nucleic Acid).

10

15 The template may comprise flanking regions. The flanking region can encompass a signal group, such as a fluorophor or a radio active group, to allow a direct detection of the presence of the complex. The flanking regions can also serve as priming sites for an amplification reaction, such as PCR. The template may in certain embodiments comprise an affinity region having the property of being able to hybridise to a building block.

20

It is to be understood that when the term template is used in the present description and claims, the template may be in the sense or the anti-sense format, i.e. the template part of the complex can be a sequence of codons

25

30

6 which actually codes for the molecule or can be a sequence complementary thereto.

The encoded molecule is formed by a variety of reactants which have reacted with each other and/or a scaffold molecule. Optionally, this reaction product may be post-modified to obtain the encoded molecule displayed on the microarray. The post-modification may involve the cleavage of one or more chemical bond attaching the encoded molecule to the template in order more efficiently to display the encoded molecule.

10 The formation of an encoded molecule generally starts by a scaffold, i.e. a chemical unit having one or more reactive groups capable of forming a connection to another reactive group positioned on a chemical entity, thereby generating an addition to the original scaffold. A second chemical entity may react with a reactive group also appearing on the original scaffold or a reactive group incorporated by the first chemical entity. Further chemical entities maybe involved in the formation of the final reaction product. The formation of a connection between the chemical entity and the nascent encoded molecule maybe be mediated by a bridging molecule. As an example, if the nascent encoded molecule and the chemical entity both comprises an amine group a connection between these can be mediated by a dicarboxylic acid.

The encoded molecule may be attached directly the template or through a suitable linking moiety. Furthermore, the encoded molecule may be linked to the template through a cleavable linker to release the encoded molecule at a point in time selected by the experimenter.

The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block prior to the participation in the formation of the reaction product leading the final encoded molecule. Besides the chemical entity, the building block generally comprises an anti-codon. In some embodiment the building block

7 also comprise an affinity region providing for affinity towards the nascent complex.

Thus, the chemical entities are suitably mediated to the nascent encoded molecule by a building block, which further comprises an anticodon. The anti-codon serves the function of transferring the genetic information of the building block in conjunction with the transfer of a chemical entity. The transfer of genetic information and chemical entity may occur in any order, however, it is important that a correspondence is maintained in the complex.

5 The chemical entities are preferably reacted without enzymatic interaction. Notably, the reaction of the chemical entities is preferably not mediated by ribosomes or enzymes having similar activity. The chemical entities may be reacted with each other or a scaffold having a recipient reactive group.

10 According to certain aspects of the invention the genetic information of the anti-codon is transferred by specific hybridisation to a codon on the template. Other methods for transferring the genetic information of the anti-codon to the nascent complex are to anneal an oligonucleotide complementary to the anti-codon and attach this oligonucleotide to the complex, e.g. by ligation. A still further method involves transferring the genetic information of the anti-codon to the nascent complex using a polymerase and a mixture of dNTPs.

15 The chemical entity of the building block serves the function of being a precursor for the structural entity eventually incorporated into the encoded molecule. Therefore, when it in the present application with claims is stated that a chemical entity is transferred to a nascent encoded molecule it is to understand that not necessarily all the atoms of the original chemical entity is to be found in the eventually formed encoded molecule. Also, as a consequence of the reactions involved in the connection, the structure of the chemical entity can be changed when it appears on the nascent encoded molecule. Especially, the cleavage resulting in the release of the entity may

20 25 30

8

generate a reactive group which in a subsequent step can participate in the formation of a connection between a nascent complex and a chemical entity.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The connection is facilitated by one or more reactive groups of the chemical entity. The number of reactive groups which appear on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffold capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds. A scaffold is a core structure, which forms the basis for the creation of multiple variants. The variant forms of the scaffold are typically formed through reaction of reactive groups of the scaffold with reactive groups of other building blocks, optionally mediated by fill-in groups or catalysts, under the creation of a connection between the entities. The chemical entities to be connected to the scaffold may contain one, two or several reactive groups able to form connections.

The reactive group of the building block may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection. Fig. 5 shows examples of various reactive groups and the corresponding connection formed.

9

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a reagent or and enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage. Fig. 6 shows examples of conditions for linkers between the building block and the chemical entity to be cleaved.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In general, it is preferred to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above. In fig. 4 exemplary reactive groups leading to transfer of a chemical entity to the entities harbouring one of the reactive groups are shown.

It is important for the method according to the invention that at least one linkage remains intact between the encoded molecule and the template. In case the method essentially involves the transfer of chemical entities to a scaffold or an evolving polymer, the eventually scaffolded molecule or the polymer may be attached with a selectively cleavable linker. The selectively cleavable linker is designed such that it is not cleaved under conditions which result in a transfer of the chemical entity to the nascent encoded molecule.

8

5 The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The connection is facilitated by one or more reactive groups of the chemical entity. The number of reactive groups which appear on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffold capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds. A scaffold is a core structure, which forms the basis for the creation of multiple variants. The variant forms of the scaffold are typically formed through reaction of reactive groups of the scaffold with reactive groups of other building blocks, optionally mediated by fill-in groups or catalysts, under the creation of a connection between the entities. The chemical entities to be connected to the scaffold may contain one, two or several reactive groups able to form connections.

10 The reactive group of the building block may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection. Fig. 5 shows examples of various reactive groups and the corresponding connection formed.

15 The simultaneous connection and cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above. In fig. 4 exemplary reactive groups leading to transfer of a chemical entity to the entities harbouring one of the reactive groups are shown.

20 It is important for the method according to the invention that at least one linkage remains intact between the encoded molecule and the template. In case the method essentially involves the transfer of chemical entities to a scaffold or an evolving polymer, the eventually scaffolded molecule or the polymer may be attached with a selectively cleavable linker. The selectively cleavable linker is designed such that it is not cleaved under conditions which result in a transfer of the chemical entity to the nascent encoded molecule.

9

25 The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The connection is facilitated by one or more reactive groups of the chemical entity. The number of reactive groups which appear on the chemical entity is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffold capable of being reacted further. One, two or more reactive groups intended for the formation of connections, are typically present on scaffolds. A scaffold is a core structure, which forms the basis for the creation of multiple variants. The variant forms of the scaffold are typically formed through reaction of reactive groups of the scaffold with reactive groups of other building blocks, optionally mediated by fill-in groups or catalysts, under the creation of a connection between the entities. The chemical entities to be connected to the scaffold may contain one, two or several reactive groups able to form connections.

30 The reactive group of the building block may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection. Fig. 5 shows examples of various reactive groups and the corresponding connection formed.

10 The attachment of the chemical entity can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the back bone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational spaced sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule.

15 The design of building blocks comprising the anti-codon may be aimed at obtaining annealing temperatures in a specific range for all or some of the building block:template hybrids to ensure that the anti-codons have been annealed to the template before the chemical entities are connected to each other through a chemical reaction.

20 The templates are preferably designed to have an annealing temperature within a certain range to obtain a condition at which all or at least the majority of complexes can be annealed to the probes of the array through the templates.

25 The complexes for the library can be prepared in accordance with a variety of methods. Examples of these methods are depicted below and generally described in PCT/DK/02/00419.

30 A first embodiment is based on the use of a polymerase to incorporate unnatural nucleotides as building blocks. Initially, a plurality of template oligonucleotides are provided. Subsequently a primer is annealed to the each template and a polymerase is extending the primer using nucleotide derivatives which have appended chemical entities. Subsequent to or

10 simultaneously with the incorporation of the nucleotide derivatives, the chemical entities are reacted to form a reaction product.

15 Several possible reaction approaches for the chemical entities are apparent.

20 5 First, the nucleotide derivatives can be incorporated and subsequently polymerised. In the event the chemical entities each carry two reactive groups, the chemical entities can be attached to adjacent chemical entities by a reaction of these reactive groups. Exemplary of the reactive groups are amine and carboxylic acid, which upon reaction form an amide bond.

25 10 Adjacent chemical entities can also be linked together using a linking or bridging moiety. Exemplary of this approach is the linking of two chemical entities, each bearing an amine group by a bi-carboxylic acid. Yet another approach is the use of a reactive group between a chemical entity and the nucleotide building block, such an ester or a thioester group. An adjacent building block having a reactive group such as an amine may cleave the interspaced reactive group to obtain a linkage to the chemical entity, e.g. by an amide linking group.

30 15 A second embodiment for obtainment of complexes pertains to the use of hybridisation of building blocks to a template and reaction of chemical entities attached to the building blocks in order to obtain a reaction product. This approach comprises that a plurality of templates are contacted with a plurality of building blocks, wherein each building block comprises an anti-codon and a chemical entity. The anti-codons are designed to have a recognition sequence, i.e. a codon, on the template. Subsequent to the annealing of the anti-codons and the codons to each other a reaction of the chemical entities are effected to obtain a reaction product.

35 20 The template may be associated with a scaffold. Building blocks bringing chemical entities in may be added sequentially or simultaneously and a reaction of the reactive group of the chemical entity may be effected at any time after the annealing of the building blocks to the template.

12

A third embodiment for the generation of a complex includes chemical or enzymatic ligation of building blocks when these are lined up on a template. Initially a plurality of templates are provided, each having one or more codons. The templates are contacted with building blocks comprising anti-codons linked to chemical entities. The two or more anti-codons annealed on a template are subsequently ligated to each other and a reaction of the chemical entities is effected to obtain a reaction product.

10 A fourth embodiment makes use of the extension by a polymerase of an affinity sequence of the nascent complex to transfer the anti-codon of a building block to the nascent complex. The method implies that a nascent complex comprising a scaffold and an affinity region, which may and may not contain coding regions. A building block comprising a region complementary to the affinity section is subsequently annealed to the nascent complex and the anti-codon region of the building block is transferred to the nascent complex by a polymerase. The transfer of the chemical entity may be transferred prior to, simultaneously with or subsequent to the transfer of the anti-codon.

20 After or simultaneously with the formation of the reaction product some of the linkers to the template may be cleaved, however at least one linker must be maintained to provide for the complex.

25 The library of the complexes may be added to the oligonucleotide microarray under hybridisation conditions in order for each template to anneal to a cognate probe on the microarray. The hybridisation conditions may be appropriately adjusted by a person skilled in the art taking into account the number and kind of nucleobases that participate in the formation of the hybrid.

13

It is within the capability of the skilled person in the art to construct the desired design of an oligonucleotide. When a specific annealing temperature is desired it is a standard procedure to suggest appropriate compositions of nucleic acid monomers and the length thereof. The construction of an appropriate design may be assisted by software, such as Vector NTI Suite or the public database at the Internet address <http://www.nwrfsc.noaa.gov/protocols/diligoTMcalc.html>.

5 The conditions which allow specific hybridisation of the templates and probes are influenced by a number of factors including temperature, salt concentration, type of buffer, and acidity. It is within the capabilities of the person skilled in the art to select appropriate conditions to ensure that the contacting between the templates and the probes are performed at hybridisation conditions. The temperature at which two single stranded oligonucleotides forms a duplex is referred to as the annealing temperature or the melting temperature. The melting curve is usually not sharp indicating that the annealing occurs over a temperature range. The second derivative of the melting curve is used herein to indicate the annealing temperature.

10 The array according to the present invention may have many uses. One aspect of the present invention relates to methods for detecting the presence or absence of, and/or measuring the amount of target molecules in a sample, wherein the method employs complexes comprising encoded molecules, which is attached in an array system. The target molecule in the sample which has an affinity towards the encoded molecule immobilised on the microarray will bind to the encoded molecule thus making the detection or measuring possible. In the event a variety of target molecules are present in the sample and a variety of encoded molecules are presented, multiple determinations are rendered possible.

15 20 25 30 The invention described herein provides arrays that can measure different amounts at the protein level without the use of proteins or peptides as detection molecules. The template-displaying molecule technology could be used

14

to identify small molecules binding to numerous targets. These binding molecules could be arrayed in specific positions and work as detection molecules to measure the amount of various biomarkers. For example, binding molecule against cytokines or enzymes known to be involved in a specific pathway could be generated with the describe technology. These binding molecules could then be attached in an array format to be used to measure the absolute or relative amount of each cytokine or enzyme.

The template-encoded molecule complexes can be directly applied to a pre-spotted DNA and hybridised the probe, optionally using an adapter oligonucleotide, which is complementary to the probe as well as the template. Another possibility is that the synthesis could be performed directly on the pre-coated template using a polymerase and the nucleotides analogues. Making addressable microarrays with this technology will lead to high-throughput deposition of thousands of different functional molecules onto different locations of a chip. The overall principal is shown in figure 1.

Ordered display of encoded molecules is a powerful tool for the identification of previous unknown target-encoded molecule interaction. In one specific format a target is detectably labelled, e.g. with a fluorescent dye, and incubated with a microarray displaying encoded molecules. By this approach, the identity of encoded molecules that are able to bind to a target molecule are determined from the location of the spots on the microarray that becomes labelled due to binding of the target. Related targets can be compared in order to identify molecules with high specificity for a specific subtype by analysis the binding pattern of these related targets on identical arrays of molecules. The above specific format may find application in the drug discovery process in which validated targets are used in order to identify suitable binding molecules.

15

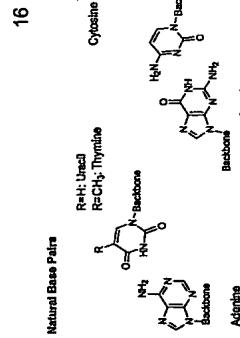
Nucleotides

The nucleotides used in the present invention may be linked together in an oligonucleotide. Each nucleotide monomer is normally composed of two parts, namely a nucleobase moiety, and a backbone. The backbone may in some cases be subdivided into a sugar moiety and an internucleoside linker.

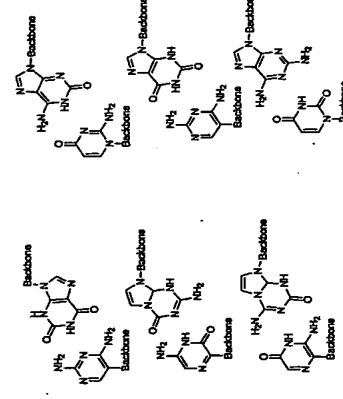
The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N⁸-methyladenine, 7-deazaxanthine, 7-deazaguanine, N⁴,N¹-ethanocytosin, N⁶,N⁶-ethano-2,6-diamino-purine, 5-methylcytosine, 5-(C³-C⁶)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudouracil, pseudouracil, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat. No. 5,432,272. The term "nucleobase" is intended to cover these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, 5-methylcytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

Examples of suitable specific pairs of nucleobases are shown below:

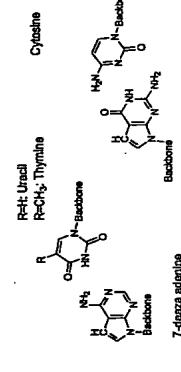
25



Synthetic purine bases pairing with natural pyrimidines



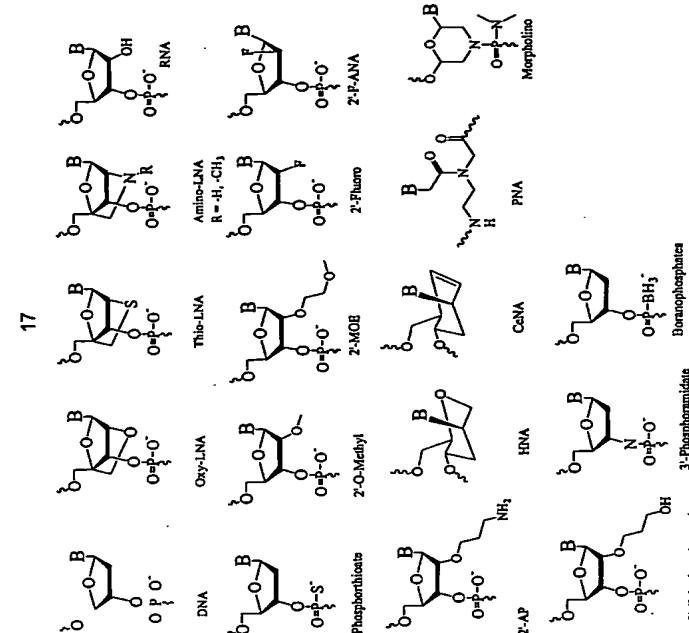
Synthetic purine bases pairing with natural pyrimidines



7-deaza adenine

7-deaza guanine

Suitable examples of backbone units are shown below (B denotes a nucleobase):



The sugar moiety of the backbone is suitably a pentose but may be the appropriate part of an PNA or a six-member ring. Suitable examples of possible pentoses include ribose, 2'-deoxyribose, 2'-O-methyl-ribose, 2'-furan-ribose, and 2'-4-O-methyl-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity.

An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer when the sugar moiety of the backbone is a pentose, like ribose or 2'-deoxyribose. The internucleoside linkage may be the natural occurring phosphodiester linkage or a derivative thereof. Examples of such derivatives include phosphothioate, methylphosphonate,

10

5

18

phosphoramidate, phosphotriester, and phosphodiesterate. Furthermore, the internucleoside linker can be any of a number of non-phosphorous-containing linkers known in the art.

Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine. Inosine is a non-specific pairing nucleoside and may be used as a universal base as discussed above because inosine can pair nearly isoenzymatically with A, T, and C.

Brief Description of the Figures

15 Fig. 1 shows an array of displayed encoded molecules.

Fig. 2 shows the synthesis of an array of encoded molecules.

Fig. 3 shows the selection to obtain encoded molecules for array analysis

Fig. 4 shows reaction types allowing simultaneous reaction and cleavage, i.e. transfer of chemical entities to a nascent encoded molecule.

20 Fig. 5 shows pairs of reactive groups and the resulting bond.

Fig. 6 shows various cleavable chemical bonds, the products obtained and the agents necessary for the cleavage to occur.

Detailed description of the figures

25 Fig. 1 shows an encoded molecule chip. A DNA library is spotted in array format on a suitable surface. A library of complexes comprising single-stranded template DNA is added and allowed to hybridise to the complementary DNA strand. This allows site-specific immobilization of the encoded molecules. The site-specific immobilization is controlled by the codons of the complex.

19

Fig. 2 shows the hybridization of encoded molecules mediated by the codon composition in each template molecule. According to a specific use, a biological sample containing target molecules is added and non-bound material is washed off. The final step is the detection of bound material in each single spot. In this example, the target molecule is labelled for detection.

5

Fig. 3 shows the selection of an initial encoded library against a target. The enriched encoded molecules are hybridized on the array due to the precise combination of codons in the template molecule. Finally, target molecule(s) are allowed to bind to the arrayed oligonucleotides and detected.

10

Fig. 4 shows different classes of reactions which mediate transfer of a chemical entity from a building block to another entity, or to an anchorage point. For simplicity reasons only, the receiving entity is shown as a building block; it is to be understood that the receiving entity can be covalently attached to the template as well. The reactions have been grouped into three different classes: Nucleophilic substitutions, addition-elimination reactions, and transition metal catalyzed reactions.

(A) Reaction of nucleophiles with carbonyls. As a result of the nucleophilic substitution, the functional group R is translocated to the monomer building block initially carrying the nucleophile.

(B) Nucleophilic attack by the amine on the thioester leads to formation of an amide bond, in effect translocating the functional group R of the thioester to the other monomer building block.

25 (C) Reaction between hydrazine and β -ketoester leads to formation of pyrazolone, in effect translocating the R and R' functional groups to the other monomer building block.

(D) Reaction of hydroxylamine with β -ketoester leads to formation of the isoxazolone, thereby translocating the R and R' groups to the other monomer building block.

30

20

(E) Reaction of thiourea with β -ketoester leads to formation of the pyrimidine, thereby translocating the R and R' groups to the other monomer building block.

(F) Reaction of urea with malonate leads to formation of pyrimidine, thereby translocating the R group to the other monomer building block.

(G) Depending on whether Z = O or Z = NH, a Heck reaction followed by a nucleophilic substitution leads to formation of coumarin or quinolinon, thereby translocating the R and R' groups to the other monomer building block.

(H) Reaction of hydrazine and phthalimides leads to formation of phthal-hydrazone, thereby translocating the R and R' groups to the other monomer building block.

(I) Reaction of amino acid esters leads to formation of diketopiperazine, thereby translocating the R group to the other monomer building block.

(J) Reaction of urea with α -substituted esters leads to formation of hydrazo- toin, and translocation of the R and R' groups to the other monomer building block.

(K) Alkylation may be achieved by reaction of various nucleophiles with sulfonates. This translocates the functional groups R and R' to the other monomer building block.

(L) Reaction of a di-activated alkene containing an electron withdrawing and a leaving group, whereby the alkene is translocated to the nucleophile.

(M) Reaction of disulfide with mercaptane leads to formation of a disulfide, thereby translocating the R' group to the other monomer building block.

(N) Reaction of amino acid esters and amino ketones leads to formation of benzodiazepinone, thereby translocating the R group to the other monomer building block.

21

(O) Reaction of phosphonates with aldehydes or ketones leads to formation of substituted alkenes, thereby translocating the R" group to the other monomer building block.

(P) Reaction of boronates with aryls or heteroaryl is results in transfer of an aryl group to the other monomer building block (to form a bialy).

(Q) Reaction arylsulfonates with boronates leads to transfer of the aryl group.

(R) Reaction of boronates with vinyls (or alkynes) results in transfer of an aryl group to the other monomer building block to form a vinyl/arene (or alkynyl/arene).

(S) Reaction between aliphatic boronates and arylhalides, whereby the aryl group is translocated to yield an alkyl/arene.

(T) Transition metal catalysed alpha-alkylation through reaction between an enolether and an arylhalide, thereby translocating the aliphatic part.

(U) Condensations between e.g. enamines or enolethers with aldehydes leading to formation of alpha-hydroxy carbonyls or alpha,beta-unsaturated carbonyls. The reaction translocates the nucleophilic part.

(V) Alkylation of alkylhalides by e.g. enamines or enolethers. The reaction translocates the nucleophilic part.

(W) [2+4] cycloadditions, translocating the diene-part.

(X) [2+4] cycloadditions, translocating the ene-part.

(Y) [3+2] cycloadditions between azides and alkenes, leading to triazoles by translocation of the ene-part.

(Z) [3+2] cycloadditions between nitriloxides and alkenes, leading to isoxazoles by translocation of the ene-part.

Fig. 5 shows collection of reactive groups that may be used for templated synthesis, along with the bonds formed upon their reaction. After reaction, activation (cleavage) may be required.

30

22

Fig. 6 shows various cleavable linkers, the conditions for their cleavage, and the resulting products.

Example

Example 1. An encoded molecule library of small molecules displayed on an array

This example describes how small molecules from an encoded library can be positioned on an array. These libraries are encoded by codons that codes for each chemical entity in the final displayed molecule. These codons describe the synthetic history which is directed by the template. This example will show how the codons can be used to positioning the displayed molecules on an array in a predetermined way through the codon composition in each individual template molecule.

The example below shows the encoding process of a 27-membered library including the RGD (Arg-Gly-Asp) sequence. This tri-peptide sequence is known to bind to various integrins such as the $\alpha_4\beta_3$ Integrin, for example. The library has 27 different members because of the total amount of combinations possible for a tri-peptide (3^3).

The scheme below shows the encoding of the RGD sequence as an example of all possible combination of these three different chemical entities. The annealing of the identifier (upper strand) to the building block (lower strand) allows the transfer of the chemical entities and their corresponding anti-codons to the nascent complex. The chemical entities are transferred by a chemical reaction and the information of the anti-codon are transferred to the nascent complex by extending the identifier using a polymerase and a mixture of dNTPs. The letters in bold indicate a flanking region of the anti-codon and the anticodon as well as the codon is underlined. I indicate the non-discriminating base inosine.

30

STEP 1A. Annealing

-**C**A CAC TAG CTT GAG CAC AC

23

-b-CAT GCG ATC GAA CTC GTC RG GRATCTCTAGCTAC

STEP 1B. Transfer of chemical entity D and anti-codon

-D-GCA GAC TAG CTT GAG CAC AC CATGGAGCTCCGAC
-G-CAT GCG ATC GAA CTC GTC RG TO GRATCTCTAGCTAC

STEP 2A. Annealing

-D-GCA GAC TAG CTT GAG CAC AC CATGGAGCTCCGAC
-G-CAT GCG ATC GAA CTC GTC RG TO GRATCTCTAGCTAC

10

STEP 2B. Transfer of chemical entity G and anti-codon.

-G-D-GCA CAC TAG CTT GAG CAC AC CATGGAGCTCCGAC
-CCT GCG ATC GAA CTC GTC RG TO GRATCTCTAGCTAC

15

STEP 3A. Annealing

-G-D-GCA CAC TAG CTT GAG CAC AC CATGGAGCTCCGAC
-CCT GCG ATC GAA CTC GTC RG TO GRATCTCTAGCTAC

20

STEP 3B. Transfer of chemical entity D and anti-codon.

-R-G-D-GCA CAC TAG CTT GAG CAC AC CATGGAGCTCCGAC
-TCG CCT GCG ATC GAA CTC GTC RG TO GRATCTCTAGCTAC

AGG GCG

AGG GCG

25

STEP 3B. Transfer of chemical entity D and anti-codon.

-R-G-D-GCA CAC TAG CTT GAG CAC AC CATGGAGCTCCGAC
-TCG CCT GCG ATC GAA CTC GTC RG TO GRATCTCTAGCTAC

AGG GCG

AGG GCG

The transfer of chemical entities is described in detail below. This scheme shows in the first step how D is transferred from a building block to the identifier molecule and then how G and R is transfer from the building block to the scaffold (an amine group). The final step is a deprotection to obtain the RGD peptide linked to the template. The final product is a library complexes, wherein each complex comprises a template and an encoded tri-peptide.

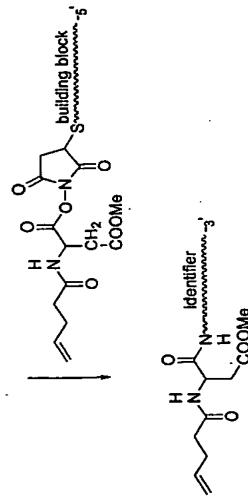
30

STEP 1A. Annealing

-**C**A CAC TAG CTT GAG CAC AC

24

Identifier
H₂N~~~~~³

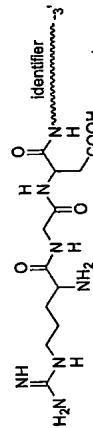


卷之三

1) 1a. THF: H₂O (1:1) 30 min

2)

3) I_2 , THF:H₂O (1:1), 30 min
 4) pH = 11.8



25

The small molecule binding setup. In this example, the array setup was used in a regular ELISA assay to confirm the binding of integrin to the immobilized RGD-template molecule. The ELISA was performed by immobilizing the template molecules using biotin binding to immobilized streptavidin. The component involved in this setup is shown below. The binding of integrin to the immobilized RGD-template molecule was detected using a specific antibody.

5

Transfer of protected G

building block

2) $\xrightarrow{\hspace{1cm}}$ identifier

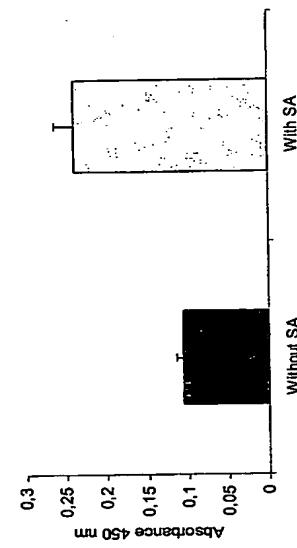
Streptavidin

The result of the ELISA is shown below. The result shows that integrin binding is dependent of the immobilized RGD-template molecule. This setup with the immobilized RGD-template molecule is identical to the situation on the array except that the template molecule is immobilized through the probe that is complementary to the template molecule.

15

26

Integrin Elisa



The ELISA was performed using a 20-mer 5' biotinylated and 3' cRGD peptide coupled oligos. This construct were bound to precoated streptavidin 8 wall strips (Pierce cat #5120) blocked over night in 0.5% Tween 20 (Sigma cat # P-9416), 3% casein (Sigma cat# C-8654) and 0.1 mg/ml herring sperm (Sigma cat# D-3159) in PBS .3 pmol oligo/well diluted in 100 μ l wash buffer (0.5% Tween 20 , 3% casein in PBS) 1 hour shaking at r.t. After washing 5 times, block buffer was added and incubated another hour at r.t shaking. 0.1 μ g/well α v β 3 human purified Integrin (Belkin V.M et al (1990) J.Cell Biol. 111: 2159-2170) (Chemicon cat # CC1018) was added to each well in 100 μ l wash buffer containing 1mM MnCl₂ and incubated at room temperature shaking for 2 hours. After washing away unbound Integrin, α v β 3 Integrin mouse monoclonal primary antibody (abcam cat # Ab7167) previously described (Martini-Padura I., et al (1995) J. Path. 175: 51) was added 0.05 μ g/well in 100 μ l wash buffer, incubated shaking at r.t for 1 hour. Followed by 10 washes and subsequently 2nd polyclonal to mouse IgG horseradish peroxidase conjugated antibody (abcam cat # ab6728) incubation, 100 μ l 1:2000 dilutions in wash-buffer, 1 hour shaking at r.t. After washing bound 2nd antibody was detected by TMB plus substrate (Kem-En-Tech cat #4390L), 100 μ l added and incubated until sufficient color development, to which 100 μ l 0.2M

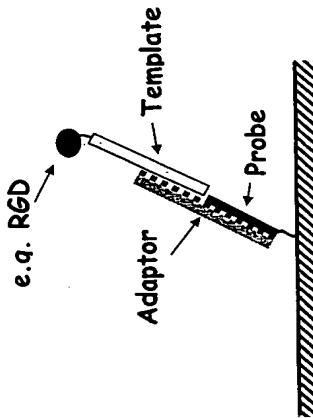
27

H₂SO₄ was added to stop the reaction. Absorbance was measured and recorded at 450 nm. Control used for the experiment was a none binding commonly used RAD peptide coupled to an oligo (J Control Release 2002 Oct 4:83(2):241-51).

5

Identification of encoded library members using arrays. GenFlex from Affymetrix is used to designing so called adaptor oligos which are complementary to the different probe oligos on the array. The adaptor oligos are also complementary to all the possible variations of codons on the identifier molecule as well as a flanking sequence showing the position of the codons. The figure below shows the outline of the setup with the adaptor oligo binding to both the identifier molecule as well as to the probe oligo on the array. This will display the molecule on the array determined by the codons in the template.

15



20 The generation of an encoded library gives a theoretical possibility of 27 different variants. To efficiently enable identification of all possible tripeptide variations generated by utilizing DNA array; codons were as described designed for which amino acid it is encoding. Flanking sequences of three nu-

mers and subsequently 2nd polyclonal to mouse IgG horseradish peroxidase conjugated antibody (abcam cat # ab6728) incubation, 100 μ l 1:2000 dilutions in wash-buffer, 1 hour shaking at r.t. After washing bound 2nd antibody was detected by TMB plus substrate (Kem-En-Tech cat #4390L), 100 μ l added and incubated until sufficient color development, to which 100 μ l 0.2M

20 The generation of an encoded library gives a theoretical possibility of 27 different variants. To efficiently enable identification of all possible tripeptide variations generated by utilizing DNA array; codons were as described designed for which amino acid it is encoding. Flanking sequences of three nu-

83

cleotides around every peptide codon was added to ensure the precise binding between the template and adaptor molecule. In order to detect all possible variants in this RGD library, 27 different adaptor oligos has to be designed that recognizes each individual template molecule. These adaptor oligos will bind specifically to the probes on the array and therefore permit the binding of the template-displayed molecules. The adaptor oligos were designed to be complementary to probes on the GenFlex from Affymetrix. This chip contains 2000 different probes, of 20-mers bound to the chip. These 27 different adaptor oligos are shown below, where the bold letters is the complementary sequence to the various probes on the chip and the normal letter sequence is the part that corresponds to the codons and flanking sequences described above.

5 RCCCTAGTG G₁ G₂ G₃ G₄ G₅ D₁ D₂ D₃ D₄ D₅

21

20

Adaptor oligos to be used for DNA array analysis for all RGD-illitary codon combinations (Marked in bold, complementary sequence to probe sequence with the probe set number on the chip to the right).

Primer # Sequence 5'-3' Probe set # on chip

1. GCT AGG CTA ATG TCG GGC TAG TAG TAG GAG ATCA CTA CGG AGA TCA CAC GGG AGA TCA CGG GT

00109

2. AGG CAG ACA ACT CAA TCC GGG TAC TTC GAC AAT ACC TTC GAC AAA CGC TTC GAC AAC GT

00348

3. TCA GAC TAG GGT AGC GCA TAG TAA GGA CTC CCT ACA GGA CTC CCA CGA GGA CTC CCC GT

00445

4. TGT CCA GTA GCT TGA GAG TCG TAG GAG ATC ACT ACC TTC GAC AAA TGC TTC GAC AAC GT

00470

5. CGA CAA GGC ATT CAC ACT AGG TAG GAG ATC ACT ACG GAG ATC ACA CSC TTC GAC AAC GT

00551

6. TCG GCG TTA CGT GGT GAC TAG TAG GAG ATC ACT ACA GGA CTC CCA CGA GGA CTC CCC GT

00684

7. CGG CAG CAA GCA GGT ATC GAG AAG TAG GAG ATC ACT ACG GAG ATC ACA CGA GGA CTC CCC GT

25

30

35

40

cleotides around every peptide codon was added to ensure the precise binding between the template and adaptor molecule. In order to detect all possible variants in this RGD library, 27 different adaptor oligos has to be designed that recognizes each individual template molecule. These adaptor oligos will bind specifically to the probes on the array and therefore permit the binding of the template-displayed molecules. The adaptor oligos were designed to be complementary to probes on the GenFlex from Affymetrix. This chip contains 2000 different probes, of 20-mers bound to the chip. These 27 different adaptor oligos are shown below, where the bold letters is the complementary sequence to the various probes on the chip and the normal letter sequence is the part that corresponds to the codons and flanking sequences described above.

5 RCCCTAGTG G₁ G₂ G₃ G₄ G₅ D₁ D₂ D₃ D₄ D₅

21

20

25

30

1. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
2. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
3. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
4. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
5. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
6. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
7. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
8. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
9. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
10. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
11. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
12. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
13. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA
14. CATTCTCTAGTCATGCTCTATGCTTCCTGCTCTATGCTGCA

8. CGA AAG CAT AAT AEG GGT GCG TAC TTC GAC AAT ACG CGG GATC ACA CGA CGA CTC CCC CCC GT
00729

9. GTA CGT TGA CAG TGT TGA CGA CGG TAC TCT GAC AAT ACG GAG ATC ACA CGG GAG ATC ACC GT
5 00852

10. TCG TCG CCC TAC CTA TGA TTA TTA TAC TTC GAC AAT ACC TTC GAC AAA CGG GAG ATC ACC GT
00891

11. ACC AAT GCA AAT AGG CGG CGG TAA CGG CTC CCT ACC TTC GAC AAA CGG GAG ATC ACC GT
10 00922

12. TCA CGC CCA CGT AGC GTT ATG TAA CGG CTC CCT ACC GAG ATC ACA CGG GAG ATC ACC GT
01033

13. GAA CTA TGC TGA CGG TAC CGG TAC TTC GAC AAT ACA CGG CTC CGA CGG GAG ATC ACC GT
15 01081

14. CCC AGG GCA AGC GAT CAT AAG TAC TTC GAC AAT ACA CGG CTC CGA CGA GEA CTC CCC CGT
20 01109

15. TCA CGT AAT TTG TTA CGG CGG TAC TTC GAC AAT ACC TTC GAC AAA CGA CGA CTC CCC CGT
01167

16. TAC CTG GCA TGA CGC GAT ATG TAA CGG CTC CCT ACC TTC GAC AAA CGA CGA CTC CCC CGT
25 01180

17. TCG AGG CTC CGA GAT GCT ATG TAA CGG CTC CCT ACC GGA CTC CGC TTC GAC AAC GT
01414

18. TGA CGG TTA GAG CCT TGT CGG TAA CGG TAC ACT ACC TTC GAC AAA CGA CGA CTC CCC CGT
30 01522

19. TCT CGG TTA CGT CGG TAA CGA CTC CCT ACC TTC GAC AAA CGG GAG ATC ACC GT
35 01528

20. CGA CGA CGA CGA ATT CGA CGA CGT ACC CGC TCC CCT ACC TTC GAC AAA CGC TTC GAC AAC GT
01548

21. GTT AGA TCA TAG TCA CGG CGG TAA CGA CTC CCT ACA CGA CGC CGA CGG GAG ATC ACC GT
40 01610

22. CAC TAA GAC ATG CAC AGC GGG TAG GAG ATC ACT ACA CGA CTC CGA CGC TTC GAC AAC GT
45 01715

23. CTA CTG ACA CTG ACC AGG GAG TAC TTC GAC AAT AGC GAG ATC ACA CGC TTC GAC AAC GT
01800

30

31

24. GCA TAC AGG TTA CGA CGC CCTG TAA CGA ATC ACT ACC TTC GAC AAA CGG GAG ATC ACC GT
01882

25. CTT CGC GCA GCT ACA TAG ATG TAA CGA CTC CCT AGG GAG ATC ACA CGA CGA CGA CTC CCC CGT
5 01828

26. GGC ATA CTA GAG TCA CGG ATG TAC TTC GAC AAT ACA CGA CTC CGA CGC TTC CCA CGG GAC AAC GT
01985

10

15

20

25

30

35

The adaptor oligos and the templates with displayed molecules were used for analysis on GenFlex according to protocol below.

GenFlex hybridization and scanning. Prior to hybridization, the Adaptor mix (100 pM each final concentration) in a hybridization buffer (100mM MES, 1M NaCl, 20 mM EDTA, 0.01% Tween 20, 1x Denhardt's), was heated to 95°C for 5 min and subsequently to 40°C for 5 min before loading onto the Affymetrix GenFlex probe array cartridge. The probe array was then incubated for 2h at 45°C at constant rotation (60 rpm). The Adaptor mix was removed from the GenFlex cartridge, and replaced with the Template in a hybridization buffer (100mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20, 1x Denhardt's). The Template hybridisation mix was heated to 95°C for 5 min and subsequently to 40°C for 5 min before loading onto the Affymetrix GenFlex probe array cartridge and hybridised for 2h at 45°C at constant rotation (60 rpm). The washing and staining procedure was performed in the Affymetrix Fluidics Station. The probe array was exposed to 2 wash in 6xSSPE-T at 25°C followed by 12 washes in 0.5xSSPE-T at 40°C. Then, 0.1µg/well α 1 β 3 human purified integrin (Belkin V.M et al (1990) J Cell boil. 111: 2159-2170) (Charmicon cat # CC1018) was added to the chip in wash buffer containing 1mM MnCl₂ and incubated at room temperature shaking for 2 hours. After washing away unbound integrin, α 1 β 3 integrin mouse monoclonal primary antibody (abcam cat

32

Ab7167) previously described (Martin-Padura I., et al (1995) J. Path. 175: 51) was added in wash buffer, and rotated at room temperature for 1 hour. Then, after 5-10 washes a 2° polydonal to mouse IgG horseradish peroxidase conjugated antibody (abcam cat # ab6728) was incubation in the wash buffer. After washing off non-bound 2° antibody the probe arrays were 5 scanned at 560 nm using a confocal laser-scanning microscope with an argon ion laser as the excitation source (Hewlett Packard GeneArray Scanner G2500A).

33

Claims

1. A microarray comprising a plurality of single stranded nucleic acid probes immobilized in discrete areas of a solid support, said probes being hybridised to a library of complexes, wherein each complex comprises an encoded molecule and a template which codes for said molecule, said template comprising a number of codons which codes for chemical entities which upon reaction form a reaction product which at least partly form part of the encoded molecule.
- 5 2. A microarray according to claim 1, wherein the chemical entities are precursors for a structural unit appearing in the encoded molecule.
3. A microarray according to claim 1 or 2, wherein the chemical entities are transferred to the nascent encoded molecule by a building block, which further comprises an anti-codon.
- 10 4. A microarray according to claim 3, wherein the information of the anti-codon is transferred in conjunction with the chemical entity to the nascent complex.
- 15 5. A microarray according to any of the claims 1 to 4, wherein the chemical entities are reacted without enzymatic interaction.
- 20 6. A microarray according to any of the claims 1 to 5, wherein the template comprises two or more codons.
- 25 7. A microarray according to any of the claims 1 to 6, wherein the nucleic acid probe of the array is hybridised to a template through an adapter oligonucleotide having a sequence complementing the probe as well as the template.
- 30 8. A method for preparing a microarray displaying a library of encoded molecules, wherein an oligonucleotide microarray comprising a plurality of single stranded nucleic acid probes immobilized in discrete areas of a solid support is mixed under conditions which allows for specific hybridisation with a library of complexes, each of said complexes comprising an encoded molecule and a template which codes for said molecule, said template comprising a number of codons which codes for

34

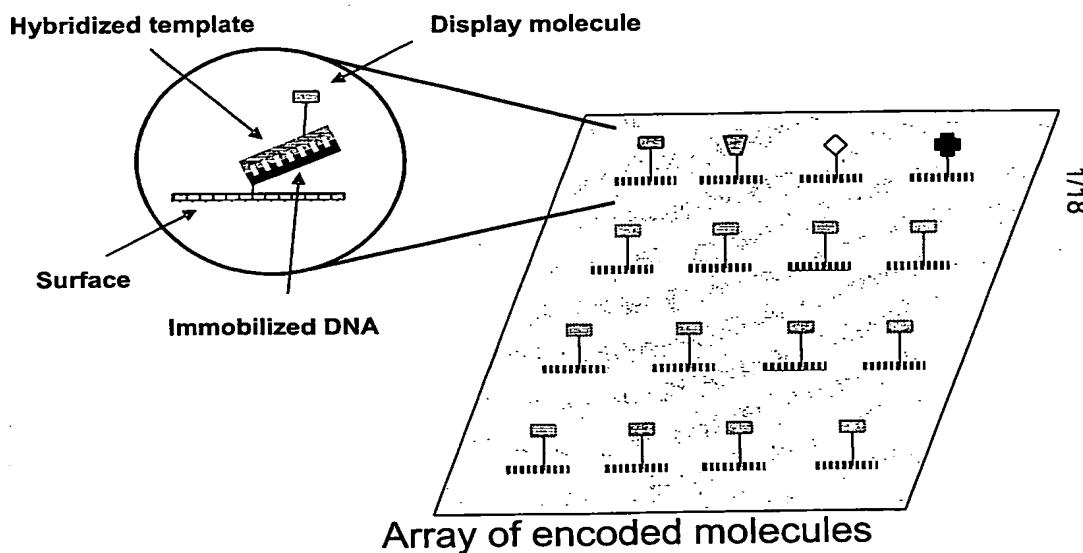
chemical entities which upon reaction form a reaction product which at least partly form part of the encoded molecule.

9. A method for identifying an encoded molecule having a preselected property, comprising the steps of

- 5 i) providing the microarray according to claim 1
- ii) adding a biological sample containing target molecules,
- iii) washing non-bound material off, and
- iv) detecting any bound material in each spot.

10. Use of a microarray according to any of the claims 1 to 7 for identifying an encoded molecule capable of binding to a target molecule.

Fig. 1



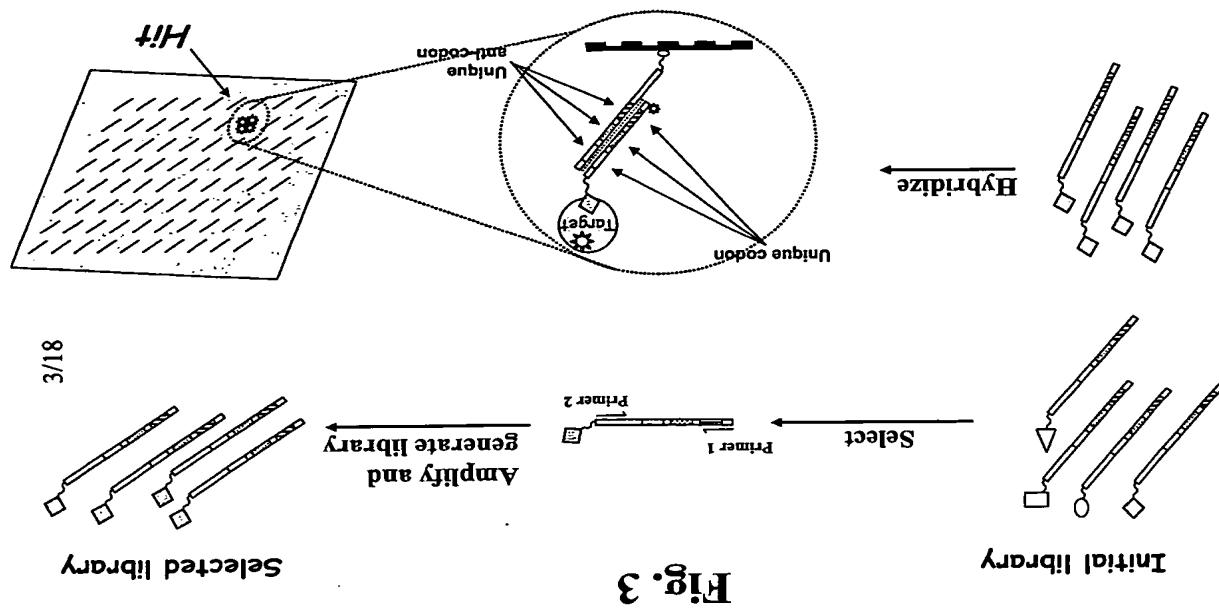


Fig. 3

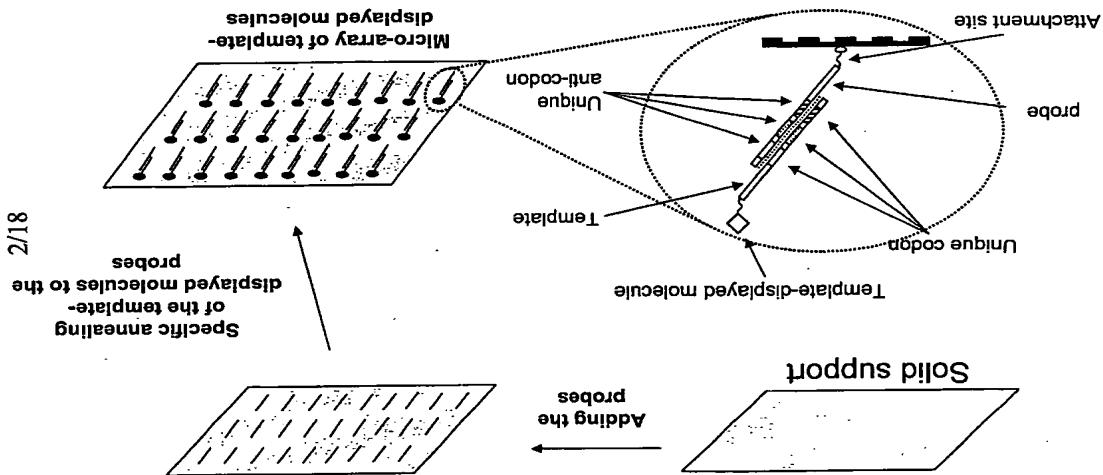
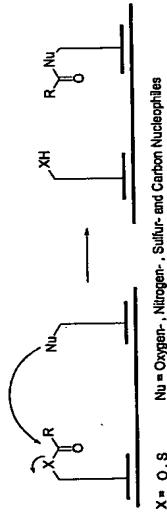
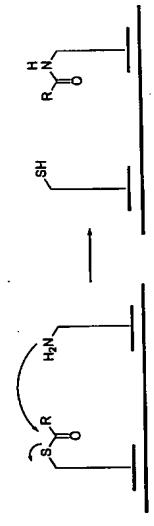
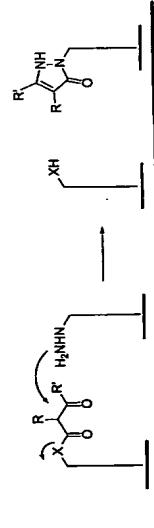


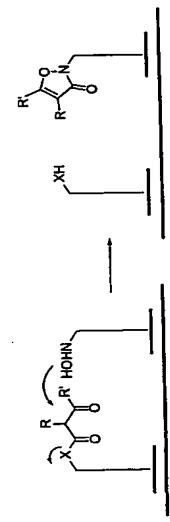
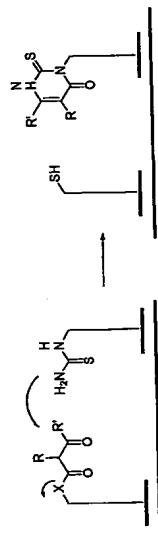
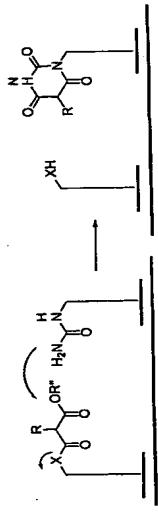
Fig. 2

4/18

Fig. 4

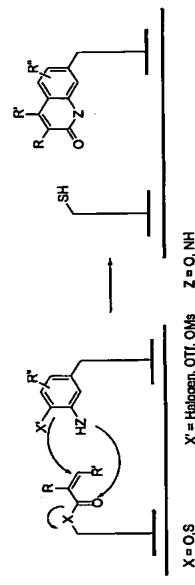
A. Acylating monomer building blocks - principle**B. Acylation****Amide formation by reaction of amines with activated esters****C. Acylation****Pyrazolone formation by reaction of hydrazines with β -Ketoesters**

5/18

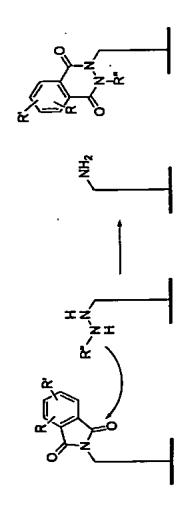
D. Acylation**Isoxazolone formation by reaction of hydroxylamines with β -Ketoesters****E. Acylation****Pyrimidine formation by reaction of thioureas with β -Ketoesters****F. Acylation****Pyrimidine formation by reaction of ureas with Malonates**

G. Acylation

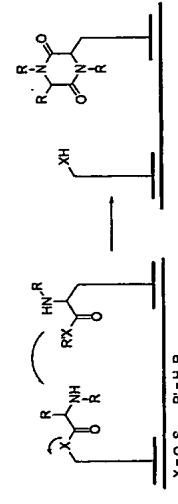
Coumarine or quinolinon formation by a Heck reaction followed by a nucleophilic substitution

**H. Acylation**

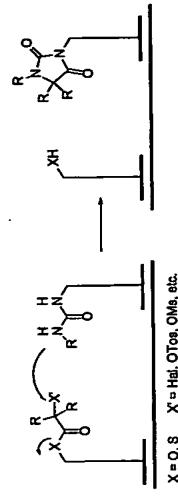
Phthalhydrazide formation by reaction of Hydrazines and Phthalimides

**I. Acylation**

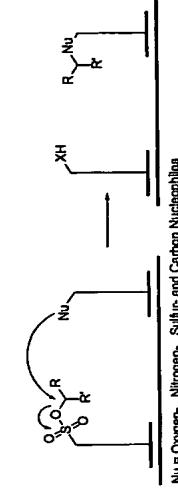
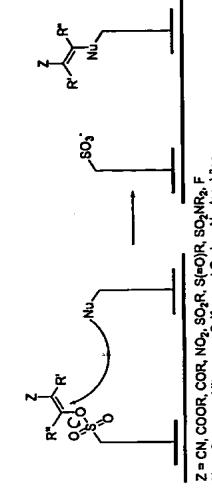
Diketopiperazine formation by reaction of Amino Acid Esters

**J. Acylation**

Hydantoin formation by reaction of Urea and α -substituted Esters

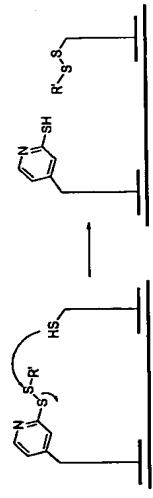
**K. Alkylation**

Alkylating monomer building blocks - principle
Alkylated compounds by reaction of Sulfonates with Nucleophiles

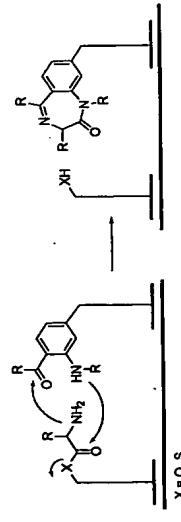
L. Vinylation monomer building blocks - principle

8/18

M. Heteroatom electrophiles
Disulfide formation by reaction of Pyridyl disulfide with
Mercaptanes

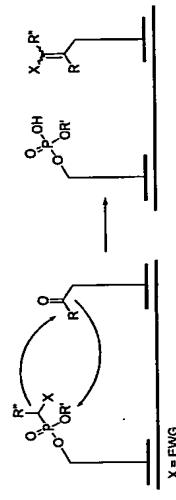


N. Acylation
Benzodiazepine formation by reaction of Amino Acid Esters and
Amino Ketones

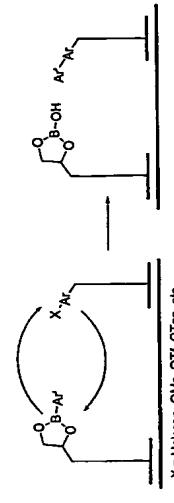


9/18

O. Wittig/Horner-Wittig-Emmons reagents
Substituted alkene formation by reaction of Phosphonates with Aldehydes or Ketones



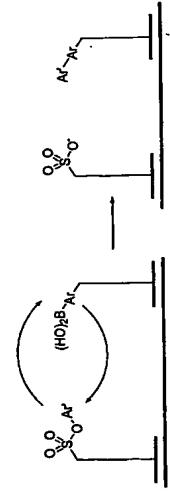
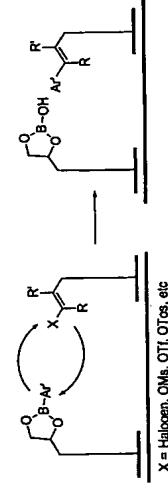
P. Arylation
Biphenyl formation by the reaction of Boronates with Aryls or
Heteroaryls



X = Halogen, OMs, OTf, OTBs, etc

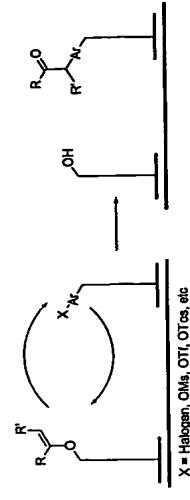
10/18

Q. Arylation
Biaryl formation by the reaction of Boronates with Aryls or Heteroaryls

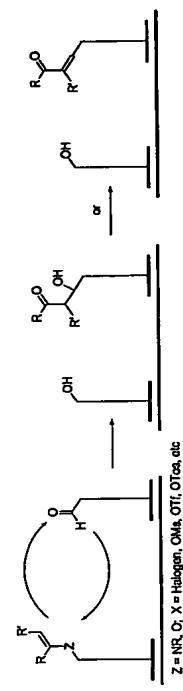
**R. Arylation****Vinyllarene formation by the reaction of alkenes with Aryls or Heteroaryls**

11/18

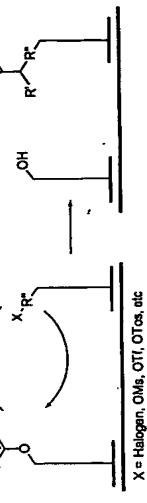
T. Alkylation
Alkylation of arenes/heteroaromatics by reaction with enol ethers



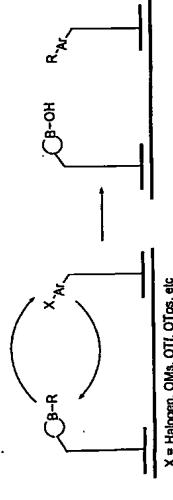
U. Condensations
Alkylation of aldehydes with enol ethers or enamines



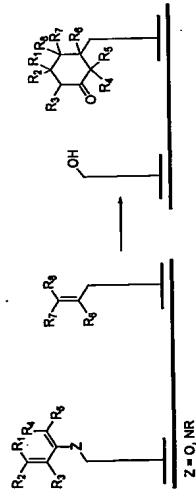
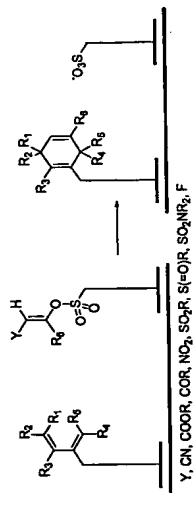
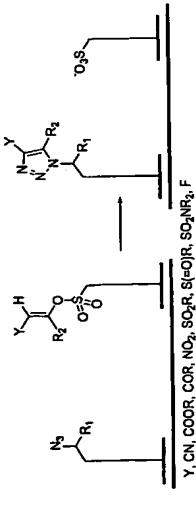
V. Alkylation
Alkylation of aliphatic halides or tosylates with enol ethers or enamines



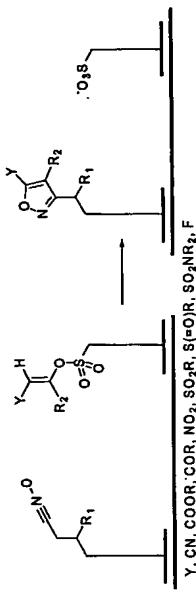
S. Alkylation
Alkylation of arenes/heteroaromatics by the reaction with Alkyl boronates



12/18

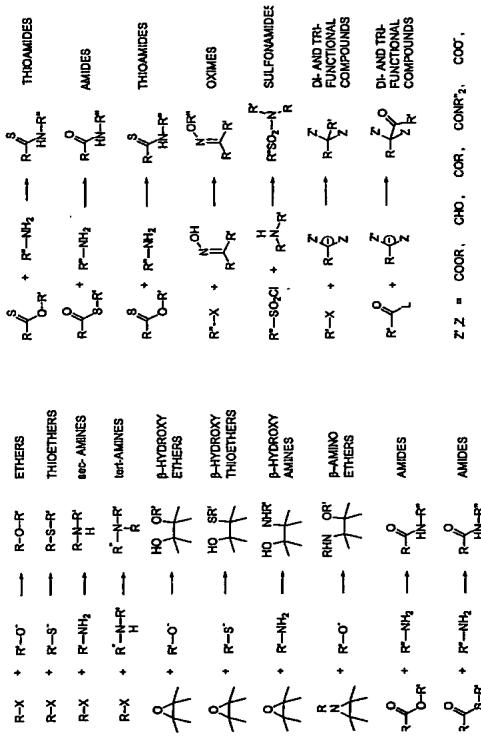
W. [2+4] Cycloadditions**X. [2+4] Cycloadditions****Y. [3+2] Cycloadditions**

13/18

Z. [3+2] Cycloadditions

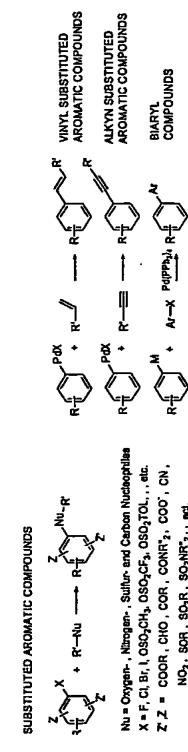
14/18

Nucleophilic substitution reactions



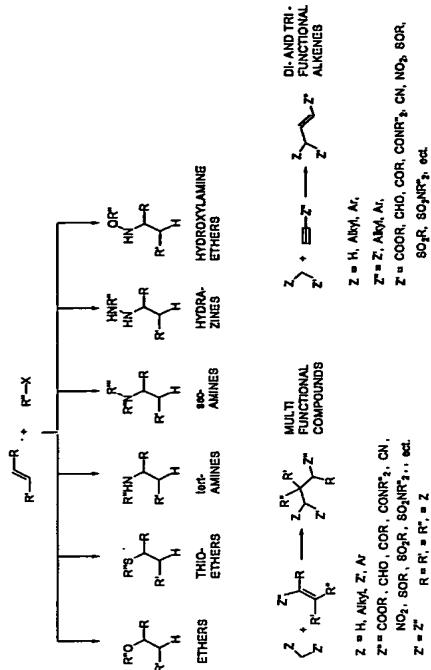
Aromatic nucleophilic substitution

Transition metal catalysed reactions

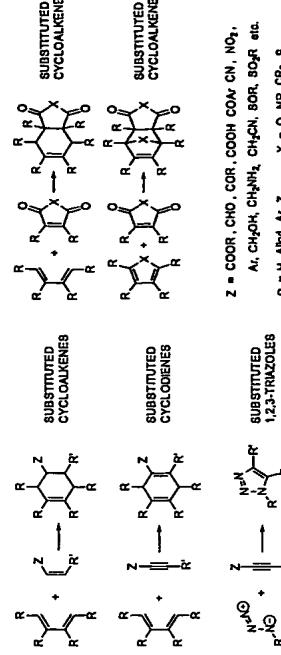


15/18

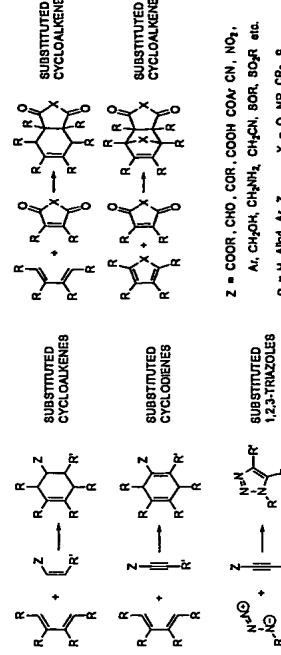
Addition to carbon-carbon multiple bonds



Case load distribution to multiple bounds



卷之三



18/18

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)



(19) World Intellectual Property Organization International Bureau

H. Linker for the formation of Ketones, Amines, Alcohols and Mercaptanes

$$\text{R}-\text{C}(=\text{O})-\text{X}-\text{R} \xrightarrow[\text{250 nm}]{\text{hv}} \text{R}-\text{C}(=\text{O})-\text{OH} + \text{H}-\text{X}-\text{R}$$

X = O, NHR, NR₂

I. Linker for the formation of Benzyl Amines, Alcohols and Mercaptanes

$$\text{R}-\text{C}(=\text{O})-\text{X}-\text{R} \xrightarrow[\text{250 nm}]{\text{hv}} \text{R}-\text{C}(=\text{O})-\text{NH}-\text{R} + \text{H}-\text{X}-\text{R}$$

X = O, R, NH, NR₂

J. Linker for the formation of Bifuryl and Bifuryl

$$\text{Ar}-\text{C}(=\text{O})-\text{X}-\text{R} \xrightarrow[\text{250 nm}]{\text{hv}} \text{Ar}-\text{C}(=\text{O})-\text{O}-\text{C}_6\text{H}_3-\text{CH}_2-\text{C}_6\text{H}_3-\text{O}-\text{C}(=\text{O})-\text{Ar} + \text{H}-\text{X}-\text{R}$$

X = O, NHR, NR₂

K. Linker for the formation of Benzyl Amines, Amines, Amines Alcohols and Phenoles

$$\text{R}-\text{C}(=\text{O})-\text{X}-\text{R} \xrightarrow[\text{250 nm}]{\text{hv}} \text{R}-\text{C}(=\text{O})-\text{NH}-\text{R} + \text{H}-\text{X}-\text{R}$$

X = O, NHR, NR₂

L. Linker for the formation of Mercaptanes

$$\text{R}-\text{C}(=\text{O})-\text{X}-\text{R} \xrightarrow[\text{250 nm}]{\text{hv}} \text{R}-\text{C}(=\text{O})-\text{SH} + \text{H}-\text{X}-\text{R}$$

X = O, NHR, NR₂

M. Linker for the formation of Glycosides

$$\text{R}-\text{C}(=\text{O})-\text{X}-\text{R} \xrightarrow[\text{250 nm}]{\text{hv}} \text{R}-\text{C}(=\text{O})-\text{O}-\text{C}_6\text{H}_3-\text{CH}_2-\text{C}_6\text{H}_3-\text{O}-\text{C}(=\text{O})-\text{R}$$

X = O, NHR, NR₂

N. Linker for the formation of Aldehydes and Glyoxylamides

$$\text{R}-\text{C}(=\text{O})-\text{X}-\text{R} \xrightarrow[\text{250 nm}]{\text{hv}} \text{R}-\text{C}(=\text{O})-\text{OH} + \text{R}-\text{C}(=\text{O})-\text{NH}-\text{R}$$

X = O, NHR, NR₂

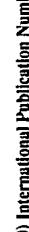
O. Linker for the formation of Aldehydes, Ketones and Aminosaccharides

$$\text{R}-\text{C}(=\text{O})-\text{X}-\text{R} \xrightarrow[\text{250 nm}]{\text{hv}} \text{R}-\text{C}(=\text{O})-\text{OH} + \text{R}-\text{C}(=\text{O})-\text{NH}-\text{R}$$

X = O, NHR, NR₂

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(10) International Publication Number



PCT

(43) International Publication Date
31 December 2003 (31.12.2003)

PCT

WO 2004/001042 A3



(88) Date of publication of the International search report:
18 March 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

<p>A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C2Q1/68 C12N15/10 //C12P21/02, C07H21/00</p> <p>According to International Patent Classification (IPC) or to both national classification and IPC</p>		<p>In - at Application No PCT/DK 03/00417</p>																
<p>B. FIELDS SEARCHED</p> <p>Minimum documentation searched (classification system followed by classification symbols)</p> <p>IPC 7 C12Q C12N C07H C12P</p>																		
<p>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched</p>																		
<p>Electronic data base consulted during the International search (name of data base and, where practical, search terms used)</p> <p>EPo-Internal, MEDLINE, BIOSIS, EMBASE</p>																		
<p>C. DOCUMENTS CONSIDERED TO BE RELEVANT</p> <table border="1"> <thead> <tr> <th>Category *</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>Y</td> <td>WO 00 23458 A (UNIV LEELAND STANFORD JUNIOR) 27 April 2000 (2000-04-27) page 1 -page 3, -line 30</td> <td>1-10</td> </tr> <tr> <td>Y</td> <td>US 5 723 598 A (BRENNER SYDNEY ET AL) 3 March 1998 (1998-03-03) column 1 ->column 3, line 1</td> <td>1-10</td> </tr> <tr> <td>Y</td> <td>SUMMERER D ET AL; "DNA-TEMPLATED SYNTHESIS: "MORE VERSATILE THAN EXPECTED" ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, VERLAG CHEMIE, WEINHEIM, DE, vol. 41, no. 1, 4 January 2002; (2002-01-04), pages 89-90, XP001170352 ISSN: 0370-0833 the whole -document</td> <td>1-10</td> </tr> <tr> <td></td> <td>-----</td> <td>-/-</td> </tr> </tbody> </table>				Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	Y	WO 00 23458 A (UNIV LEELAND STANFORD JUNIOR) 27 April 2000 (2000-04-27) page 1 -page 3, -line 30	1-10	Y	US 5 723 598 A (BRENNER SYDNEY ET AL) 3 March 1998 (1998-03-03) column 1 ->column 3, line 1	1-10	Y	SUMMERER D ET AL; "DNA-TEMPLATED SYNTHESIS: "MORE VERSATILE THAN EXPECTED" ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, VERLAG CHEMIE, WEINHEIM, DE, vol. 41, no. 1, 4 January 2002; (2002-01-04), pages 89-90, XP001170352 ISSN: 0370-0833 the whole -document	1-10		-----	-/-
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																
Y	WO 00 23458 A (UNIV LEELAND STANFORD JUNIOR) 27 April 2000 (2000-04-27) page 1 -page 3, -line 30	1-10																
Y	US 5 723 598 A (BRENNER SYDNEY ET AL) 3 March 1998 (1998-03-03) column 1 ->column 3, line 1	1-10																
Y	SUMMERER D ET AL; "DNA-TEMPLATED SYNTHESIS: "MORE VERSATILE THAN EXPECTED" ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, VERLAG CHEMIE, WEINHEIM, DE, vol. 41, no. 1, 4 January 2002; (2002-01-04), pages 89-90, XP001170352 ISSN: 0370-0833 the whole -document	1-10																
	-----	-/-																
<p><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</p> <p><input checked="" type="checkbox"/> Patent family members are listed in annex.</p>																		
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"B" earlier document but published on or after the International filing date, which may prove useful in understanding the invention</p> <p>"C" document which may throw doubt on validity of the application or which is cited to explain away the invention</p> <p>"D" document which does not relate to the invention but is cited in connection with another document referred to in the application</p> <p>"E" document relating to an oral disclosure, sees, exhibition or other means</p> <p>"F" document published prior to the International filing date but later than the priority date of the claimed</p> <p>"G" document member of the same patent family</p>																		
<p>Date of the actual completion of the International search</p> <p>3 February 2004</p>		<p>Date of mailing of the International search report</p> <p>10/02/2004</p>																
<p>Name and mailing address of the ISA</p> <p>European Patent Office, P.B. 3619 Paimiojain 2 NL - 2200 HV Fluwijk Tel. (+31-70) 340-2640, Tel. 31 65 490 011 Fax. (+31-70) 340-3018</p>		<p>Authorized officer</p> <p>Osborne, H</p>																

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

C: (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

C17/100 05/01/1999	
C1 (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT	
Category	Citation of document, with indication where appropriate, of the relevant passages
Y	BRUCK R K ET AL: "TEMPLATE-DIRECTED LIGATION OF PEPTIDES TO OLIGONUCLEOTIDES" CHEMISTRY AND BIOLOGY, CURRENT BIOLOGY, LONDON, GB, vol. 3, no. 1, January 1996 (1996-01), pages 49-56, XP0008566376 ISSN: 1074-5521 the whole document 1-10
Y	WALTER J A ET AL: "COMPLEMENTARY CARRIER PEPTIDE SYNTHESIS: GENERAL STRATEGY AND IMPLICATIONS FOR PREBIOTIC ORIGIN OF PEPTIDE SYNTHESIS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, US, vol. 76, no. 1, January 1979 (1979-01), pages 51-55, XP000857351 ISSN: 0027-8424 the whole document 1-10
Y	EP 0 778 280 A (ISIS INNOVATION) 11 June 1997 (1997-06-11) figures 4,5; example 17 1-10
Y	WO 96 41011 A (SPECTRAGEN INC) 19 December 1996 (1996-12-19) figure 3 1-10
Y	LOCKHART D J ET AL: "EXPRESSION MONITORING BY HYBRIDIZATION TO HIGH-DENSITY OLIGONUCLEOTIDE ARRAYS" BIO/TECHNOLOGY, NATURE PUBLISHING CO, NEW YORK, US, vol. 14, no. 13, 1 December 1996 (1996-12-01), pages 1675-1680, XP002022521 ISSN: 0733-222X the whole document 1-10
Y	WO 97 27317 A (CHEE MARK ; LAI CHAOQIANG (US); LEE DANNY (US); AFFYMATRIX INC (US)) 31 July 1997 (1997-07-31) figures 12-15 1-10
Y	WO 99 42605 A (SCHNABACHER ALAN W) 26 August 1999 (1999-08-26) page 2, line 11 - line 16 1-10
A	WO 94 08051 A (READER JOHN C ; STILL W CLARK (US); UNIV COLUMBIA (US); COLD SPRING) 14 April 1994 (1994-04-14) 1-10

INTERNATIONAL SEARCH REPORT

INTERACTION ON PUBLIC INFLUENCY 105

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0023458	A	27-04-2000	AU 1318400 A CA 2346989 A1 EP 1123305 A1 WO 0023458 A1	08-05-2000 27-04-2000 16-05-2001 27-04-2000
US 5723598	A	03-03-1998	US 5573905 A US 6060596 A AT 195661 T AU 685050 B2 AU 3944993 A CA 2132103 A1 DE 69328781 D1 DE 69328781 T2 DK 643778 T3 EP 0643778 A1 ES 2147197 T3 JP 7505530 T WO 9320242 A1	12-11-1996 09-05-2000 15-06-2000 15-01-1998 08-11-1993 14-10-1993 06-07-2000 26-04-2001 07-08-2000 22-03-1995 01-09-2000 22-08-1995 14-10-1993
EP 0778280	A	11-06-1997	EP 0778280 A2 AT 159167 T AT 230409 T AU 695349 B2 AU 7269194 A CA 2168010 A1 CN 1131440 A, B DE 69406544 D1 DE 69406544 T2 DE 69413967 D1 DE 711362 T3 EP 0711362 A1 ES 2108479 T3 FI 960403 A WO 9504160 A1 HU 73602 A2 JP 3289911 B2 JP 9501830 T NO 960370 A RU 2158310 C2 US 2002115691 A1 US 5770367 A US 2001031472 A1 US 6218111 B1	11-06-1997 15-11-1997 15-01-2003 13-08-1998 28-08-1995 09-08-1995 18-09-1996 04-12-1997 26-05-1998 06-02-2003 22-12-1997 15-05-1996 16-12-1997 29-01-1996 09-02-1995 30-09-1996 10-06-2002 25-02-1997 28-03-1996 27-10-2000 22-08-2002 23-06-1998 18-10-2001 17-04-2001
WO 961011	A	19-12-1996	US 5635400 A AU 718357 B2 AU 6102096 A CA 2222581 A1 CA 2332731 A1 CN 1193357 A CZ 9703926 A3 EP 0832287 A1 HU 98008910 A2 JP 11507528 T NO 975744 A PL 324000 A1 WO 712929 B2	03-06-1997 13-04-2000 30-12-1996 19-12-1996 19-12-1996 16-09-1998 17-06-1998 01-04-1998 28-07-1999 05-07-1999 05-02-1998 27-04-1998 19-12-1996 18-11-1999

INTERNATIONAL SEARCH REPORT

Information on parent family members				Int'l Application No. PCT/DK 03/00417
Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
WO 9641011 A	AU 31-07-1997	AU 4277896 A AU 7717596 A CA 2202167 A1 CZ 9700866 A3 EP 0793718 A1 EP 0931165 A1 FI 971473 A JP 10507357 T NO 971644 A US 628935 B1 WO 9713877 A1 US 6234475 B1	06-05-1996 30-04-1997 25-04-1996 17-09-1997 10-09-1997 28-07-1999 04-06-1997 21-07-1998 02-06-1997 28-08-2001 17-04-1997 22-05-2001	06-05-1996 30-04-1997 25-04-1996 17-09-1997 10-09-1997 28-07-1999 04-06-1997 21-07-1998 02-06-1997 28-08-2001 17-04-1997 22-05-2001
WO 9727317 A	31-07-1997	AU 2255397 A EP 0880598 A1 JP 2002515738 T WO 9722317 A1 US 2003180757 A1 US 2003064364 A1 US 6344316 B1 US 2002163372 A1	20-08-1997 02-12-1998 28-05-2002 31-07-1997 25-09-2003 03-04-2003 05-02-2002 07-11-2002	20-08-1997 02-12-1998 28-05-2002 31-07-1997 25-09-2003 03-04-2003 05-02-2002 07-11-2002
WO 9842605 A	26-08-1999	US 200200604 A1 AU 2871299 A CA 2322111 A1 EP 1056882 A1 JP 2002540380 T WO 9942605 A1	17-01-2002 06-09-1999 26-08-1999 06-12-2000 26-11-2002 26-08-1999	17-01-2002 06-09-1999 26-08-1999 06-12-2000 26-11-2002 26-08-1999
WO 9408051 A	14-04-1994	AT 244769 T AU 716621 B2 AU 4528897 A AU 686579 B2 AU 5536994 A CA 2143848 A1 DE 69333087 D1 DK 668897 T3 EP 0663897 A1 HU 72495 A2 IL 107166 A JP 8506175 T NO 951230 A WO 9408051 A1 US 5563324 A US 5968736 A US 5721099 A US 5789172 A US 6503759 B1	15-07-2003 02-03-2000 12-02-1998 12-02-1998 26-04-1994 14-04-1994 14-08-2003 20-10-2003 09-08-1995 28-05-1996 31-10-2000 02-07-1996 30-03-1995 14-04-1994 15-10-1996 19-10-1999 24-02-1998 04-08-1998 07-01-2003	15-07-2003 02-03-2000 12-02-1998 12-02-1998 26-04-1994 14-04-1994 14-08-2003 20-10-2003 09-08-1995 28-05-1996 31-10-2000 02-07-1996 30-03-1995 14-04-1994 15-10-1996 19-10-1999 24-02-1998 04-08-1998 07-01-2003